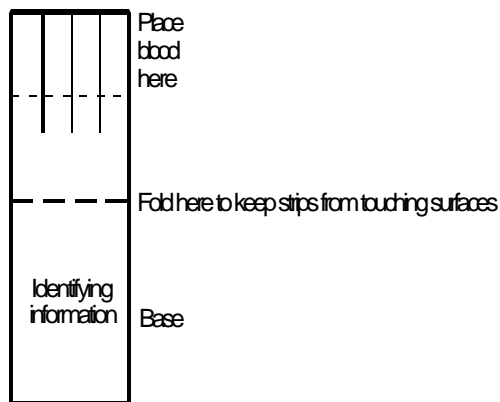


Guidelines for Protocols Involving Molecular Markers of Drug Resistance and Multiplicity of *P. falciparum* Infections

Sample collection techniques

- ? All samples will be collected on filter paper (3MM Whatman) for all the MIM/TDR projects.
- ? All samples should be clearly labelled with permanent ink with the date, study identification number, and other permanent information, e.g., post-treatment day.
- ? **Collection procedures:**
 - ? Spot blood on each of the four strips of the filter paper as shown in the figure below.
 - ? Fold the filter paper in a tent-like configuration to dry in a clean, dry container with desiccant for a minimum of three hours.



Note: Wear glove during the process and do not touch the filter paper with your bare fingers.

Sample collection times

- ? Samples should be collected on days 0, 7, 14, 21, and 28 as well as on any occasion when finger-prick blood is obtained for microscopy (e.g. during unscheduled visits for evaluation of illness).

Note: Samples should not be collected on post-treatment days (1-3), during which time the parasites are unlikely to be significantly different from day 0, unless the specific aim of the study is to assess evolution of parasites during this immediate post-treatment period.

Necessary materials:

- ? Filter paper (3MM Whatman)
- ? Scissors
- ? Small paper envelopes, plastic bags or other suitable means of storage

Procedure:

- ? Cut filter paper into rectangles approximately 2 by 5 cm.
- ? Fold in half and cut four strips approximately 0.5 by 2 cm.
- ? Blot finger-prick from one person onto four strips up to 1 cm (or more) from end. Try to get enough blood to completely saturate the paper through both sides.
- ? Allow paper to air-dry by affixing base on a tape inside a ventilated closed box. To hasten drying or protect from flies, can place in tissue culture hood, incubator or drying oven. Complete drying may prevent DNA degradation.
- ? Store and transport at room temperature in individual envelopes, or wrap in plastic. Storage in airtight plastic bag with desiccant may prevent DNA degradation.

Sample storage

- ? Sample collected should be dried, stored in the individual sealing plastic bags with desiccant pouches (silica gel) especially in areas of high humidity.
- ? Samples should be stored protected from extremes of temperature and humidity until processed for DNA extraction. When this cannot be assured under room temperature conditions, i.e., in humid environments where air-conditioning is not available, storage in a refrigerator or freezer (-20°C to -40 °C) may be considered, and great care must be taken to protect samples from frost and moisture.
- ? Extract DNA from filter paper as soon as possible.

Sample analysis

- ? Sample analysis should be based on the standardised DNA extraction, PCR and restriction digestion techniques. The Methanol and Chelex Methods are available and the expertise has been transferred to each center for DNA extraction. The RFLP-PCR and ASRA-PCR have been selected as the analytical method of choice for the network. Mutation specific PCR may be used for selected protocols by the sites and as a back up. Selection was based on the accuracy,

sensitivity, precision, and reproducibility. Primers and positive controls will be obtained from MR4. (See table 3)

DNA Extraction

Methanol fixation of blood-impregnated filter papers for PCR

- ? Cut off an approximately 3mm-square piece of blood-impregnated filter paper using a razor / pair of scissors, wiping off the razor on tissue paper between cuttings. NOTE: too much paper may inhibit PCR reactions; if parasitemia is low or the DNA is degraded, too little paper may not yield produce. If PCR doesn't work, try either larger or smaller pieces of paper.
- ? Transfer the paper to a 0.5ml PCR microfuge tube. Add approximately 50-100 ul methanol to the tube, making sure the paper is totally immersed in the methanol. Incubate for 15 minutes at room temperature.
- ? After incubation, pour out the methanol while careful to retain the paper in the tube. Pouring out the solution onto absorbent paper is helpful. Aspirate or leave the tube open on its side and allow remaining methanol to evaporate for at least one hour minutes. Make sure the paper is fully dry.
- ? To each tube containing filter paper add 50 ul water. Heat the tube for 15 minutes at 95-100°C. Occasional vortexing during the incubation is recommended to increase yield.

Use 5-10 ul of above in a 25 ul primary PCR reaction. The remainder of the extraction can be frozen for later use.

Note: 3X filter paper size could be used for extraction in 150 ul of water.

Chelex Extraction Method

- ? Dried blood-blotted filter papers (3MM Whatmann)
- ? 10% saponin in water (this can be stored at -20°C)
- ? 1X Phosphate-buffered solution PBS (pH about 7.4)
- ? 20% Chelex-100 in water (can be stored at room temperature)
- ? A heating block at 95°C

Method

1. Cut the blotted filter paper to appropriate size (3mm²) using a razor blade—the same blade can be used, wiping it on a dry tissue paper between cuttings.

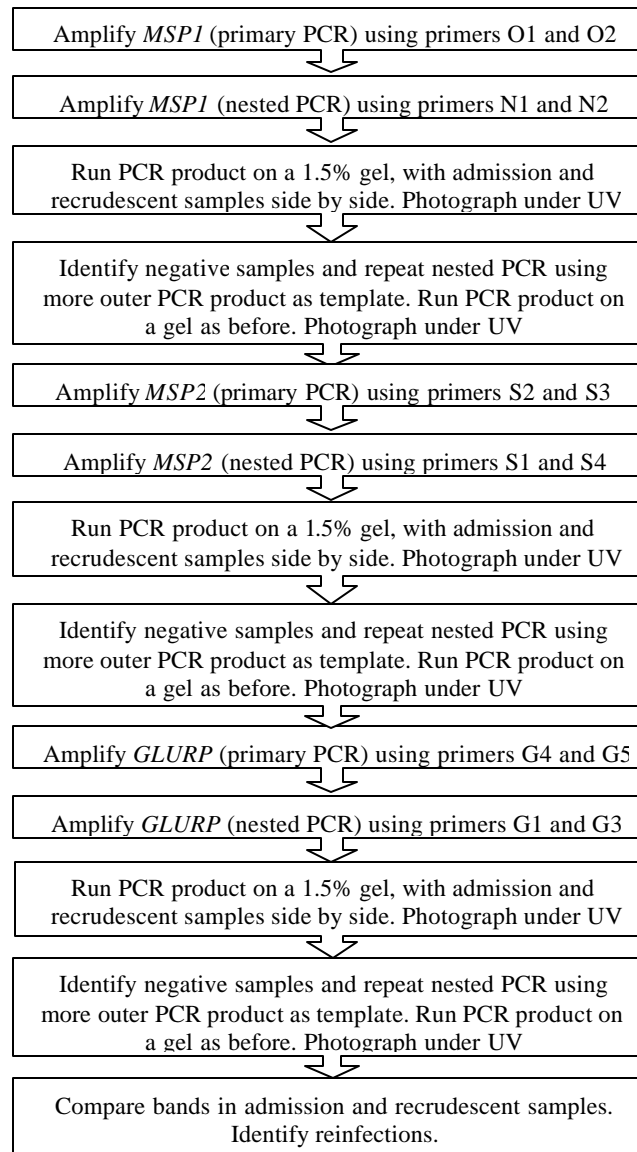
2. Combine dried, blood-blotted filter papers with 1 ml of PBS (pH 7.4) and 50 μ l of 10% saponin in 1.5 ml microfuge tubes, invert several times, and store overnight at 4°C.
 3. Microfuge tubes for 5 seconds, and aspirate the now reddish PBS/ saponin from the tubes with a clean yellow-tip attached to a pasteur pipet at the end of a vacuum assembly, using a new yellow-tip for each tube.
 4. Add 1ml of PBS/ tube (no saponin), invert several times, and incubate at 4 °C for 1-2 hours.
 5. Microfuge and aspirate as much fluid as possible, and afterwards use the yellow tip to press down filter paper into the lower third of the tube –without packing it excessively.
 6. Add 100 μ l of sterile water to each tube. Note: if blood blot is very small or very thin, and/ or if the parasitaemia is known to be low (e.g., < 1000tf), add 50 μ l instead of 100 μ l.
 7. Transfer vortexed Chelex stock solution to a 2ml microfuge tube, and using a yellow tip with its tapered end clipped off, dispense 50 μ l to each sample, vortexing or inverting the tube after every three or four transfers, i.e., be sure you are not just transferring water, with all the Chelex settled to the bottom of the dispensing tube.
 8. Extract the parasite DNA by incubating tubes for 10 minutes in a 95°C heat-block, vigorously vortexing each sample every two minutes or so throughout the incubation. It is advisable to uncap each tube after every two minutes in the block to allow for release of air-pressure, or else the tubes will “pop”.
 9. After incubation, microfuge tubes for 5 minutes at 14,000 rpm. Meanwhile, label two sets of 0.5 microfuge tubes for transfer, the second set for final storage of the extracted DNA samples.
 10. Transfer as much solution as possible from the spun tubes to the first set of microfuge tubes with a plugged 200 μ l tip, not worrying if chelex is carried over as well.
 11. Spin tubes for 10 mins, then transfer the final, white-to-yellowish supernatant, avoiding the pelleted chelex to the final set of labeled tubes. Store tubes at –20°C.
- ? Commercial and not homemade Taq polymerase, as well as digital image storage systems should be applied for all assays for comparison of data across the sites.

The protocol includes two assays for molecular markers, i.e., *(i) molecular markers for identifying antimalarial drug resistance, and (ii) molecular markers of multiplicity of P. falciparum infections (to distinguish between recrudescence and re-infection).*

Molecular Markers for Multiplicity of *P. falciparum* Infections

- ? A recurrent parasitaemia detected during follow-up is due either to *(i) a resistant parasite infection (i.e. the original infection recrudescing), or (ii) a new infection.* Comparison of parasite genotype patterns at recruitment with the pattern of the recurrent parasitaemia would distinguish between a resistant infection and a new infection.
- ? Assays for MSP1, MSP2, and GLURP should be used as standard assays to distinguish re-infected from recrudescence parasites, *until standard protocols for neutral markers such as microsatellite markers or simple sequence repeats markers that may be under immune selection are available.*

Overview of Strategy



Restriction Fragment Length Polymorphisms-PCR & Allele Specific Restriction Analysis - PCR

The methods of Duraisingh using RFLP-PCR for *dhfr* mutations will be adopted (Duraisingh *et al.*, 1998), whereas the ASRA-PCR will be used for *dhps* analysis (Kublin *et al.*, 2002).

The restriction digestions will be carried out overnight at the optimum temperature as indicated by the suppliers - New England Biolabs. The amplicon or amplified fragment will be digested without undergoing a purification process. For those reactions that require incubation of 50°C and above, mineral oil overlay is recommended.

STANDARD OPERATING PROCEDURES FOR S/P and CQ RESISTANCE MARKERS

Polymerase chain reaction followed by restriction fragment length (RFLP-PCR and ASRA-PCR Protocols

Parasites will be examined for antifolate-resistance associated point mutations in the *dhfr* (chromosome 4 -Duraisingh *et al* 1998) and *dhps* (chromosome 8) genes using a nested PCR/RFLP method described by Kublin *et al* (2002). The nested PCR approach will be used to amplify regions of *dhfr* and *dhps* genes containing antifolate associated point mutations.

Reagents

Double distilled and sterilized (PCR) water

10X PCR Buffer: 100mM Tris-Cl, pH 8.3, 500 mM KCl

dNTP mix: 10mM of each dNTP

MgCl₂: 15 mM

Primer 1

Primer 2

Taq DNA polymerase 5U/ ?l

*Mineral oil should be used where hot-bonnet is not available this step can be skipped

Nest I

Amplification cycles

Initial denaturation step at 94°C for 3mins

94°C for 1min,
50°C for 2mins,
72°C for 2 mins } 40 cycles
and final extension step at 72°C for 10mins.

Nest II

Amplification cycles

Initial denaturation step at 94°C for 2 mins

94°C for 1min,
45°C for 1min,
72°C for 2mins } 35 cycles

and a final extension step of 72°C for 10mins.

Use 5ul of nested II PCR tube are used in the digestion reactions with the appropriate buffer as described by the manufacturers (New England Biolabs Inc)

NEST I

Method: Master mix PCR

Protocol for a cocktail of 5 reactions

Final volume 25 μ l [final conc.]

2 and 3 controls (positive, negative and water) and 2 extra tubes: (Calculate the reactions for 10 tubes/ reactions)

Add reagents in the following order into a tube:

- | | |
|---|--|
| 1. H ₂ O | 14.55 μ l x 10 reactions = 145.5 μ l |
| 2. 10 x reaction buffer [15mM Mg ²⁺] | 2.5 μ l [1X] [1.5mM Mg ²⁺] x 10 = 25 μ l |
| 3. dNTP mix [2mM] | 2.5 μ l [200 μ M] x 10 = 2.5 μ l |
| 4. Primer M1 [50 μ M]
Primer M5 [50 μ M] | 0.125 μ l [0.25 μ M] x 10 = 1.25 μ l
0.125 μ l [0.25 μ M] x 10 = 1.25 μ l |
| 5. Taq DNA polymerase [5U/ μ l] | 0.2 μ l [1U/ μ l]: x 10 = 2 μ l
Mix enzyme gently and spin down briefly before use. |
| 6. Mix gently, spin down briefly | |
| 7. Aliquot 20 μ l into pre-labeled reaction tubes | Add 5 μ l of template DNA |
| 8. Overlay with 30 μ l mineral oil | |
| 9. Add 5 μ l DNA/template sample under oil | |

10. Place into a hot thermal cycler as quickly as possible

NEST II

Method: Master mix PCR

Protocol for a cocktail of 5 reactions

Final volume 25 μ l [final conc.]

2 and 3 controls (positive, negative and water) and 2 extra tubes: (Calculate the reactions for 10 tubes/reactions)

Add reagents in the following order into a tube:

- | | |
|--|--|
| 1. H ₂ O | 17.55 μ l x 10 reactions = 175.5 μ l |
| 2. 10 x reaction buffer [15mM Mg ²⁺] | 2.5 μ l (1X; [1.5mM Mg ²⁺]) x 10 = 25 μ l |
| 3. dNTP mix [2mM] | 2.5 μ l [200 μ M] x 10 = 25 μ l |
| 4. Primer M1 [50 μ M]
Primer M5 [50 μ M] | 0.125 μ l [0.25 μ M] x 10 = 1.25 μ l
0.125 μ l [0.25 μ M] x 10 = 1.25 μ l |
| 5. Taq DNA polymerase [5U/ μ l] | 0.2 μ l [1U/ μ l]: x 10 = 2 μ l
Mix enzyme gently and spin down briefly before use. |
| 6. Mix gently, spin down briefly | |
| 7. Aliquot 20 μ l into pre-labeled reaction tubes | |
| 8. Overlay with 30 μ l mineral oil | |
| 9. Add 3 μ l of water | |
| 10. Add 2 μ l primary amplicons under oil | |
| 11. Place into a hot thermal cycler as quickly as possible | |

Analysis of restriction enzymes digestion of amplified segments and control DNA is described below:

Dihydrofolate reductase dhfr

Codon-51

Amplification containing codon-51 is digested with *Tsp509 I*. If the enzyme cuts the M3-F/ fragment, a 154bp and 64bp pattern, indicative of wild type sequence (Asn-51; AAT/ AAC -51) is obtained. W2 and K1 strains of *P. falciparum* are used as the mutant and wild type controls, respectively.

Codon-59

Xmn I restriction enzyme is used to distinguish between mutant and wild type codon 59. The presence of a restriction site, allows digestion giving a 163bp, 137bp and 26bp fragments indicative of mutant (Arg-59; CGT-59). A 189b and 137b pattern, is indicative of wild type (Cys-59; TGT-59). *P. falciparum* strains K1 and T9/96 serve as mutant and wild type controls.

Codon-108

Similarly, for codon 108, the PCR products from M3-F and M4-F amplification primers, is used to detect for polymorphisms. *Alu I* restriction enzyme is used to distinguish between wild type (Ser-108; AGC-108); *BsrI* can distinguish mutant (Asn-108; AAC-108) and *BstNI* distinguish mutant variant (Thr-108; AC-108C). *Alu I* is used on fragment M4-F while *BsrI* and *BstNI* is used for M3-F. *P. falciparum* strains K1 and T9/96 serve as mutant and wild type controls for Ser108 and Asn108, respectively. Whereas strain FCR3 are used for Thr108 positive control. Fragment sizes are 180 and 146 for *BsrI* and 180bp and 145bp for *BstNI*

Codon-164

A *DraI* mismatch engineered in the F/ primer permit the distinction of the wild type (Ile-164; ATA-164) from the mutant (Leu-164; TTA) at codon-164 and using an M3-F/ amplified fragment. Two additional *DraI* sites are contained in the amplified fragment (control sites). V1/S and K1 strains of *P. falciparum* are used as mutant and wild type controls, respectively. Fragment sizes resulting are 245bp, 143bp, 107bp

Dihydropteroate synthetase, dhps

Codon-437

Polymorphism at codon 437 uses *MwoI* restriction enzyme which detects presence of wild type (Ala -437; GCT-437) while *AvaII* identifies the mutant (Gly-437; GGT-437) contained in the K-K/ PCR fragment. Mutant and wild type controls are KI and FCR3, respectively. Fragment sizes are 387bp and 41bp for *MwoI* the , 404 and 34bp for *AvaII*

Codon-540

Codon 540 wild (Lys -540; AAA-540) to mutant (Glu-540; GAA-540) changes are detected by *FokI* digestion of K-K/ PCR fragment. A digest comprising 320bp, 85bp and 33bp is indicative of Glu-540 while 405bp and 33bp is indicative of Lys-540. For mutant and wild type control, TN-1 and K1 are used respectively.

AMPLIFICATION PRODUCTS		
<u>DHFR</u>		
	Size (base pair)	
Primary PCR M1-M5	642	
Secondary PCR:		
M3-F/	522	
F-M4	326	
<u>DHPS</u>		
Primary PCR R2-R/	711	
Secondary PCR		
K-K/	438	
L-L/	161	

As a result of the very high sensitivity of the nested PCR methodology, the amount of amplification product obtained from one parasite is sufficient for visualization by ethidium bromide staining following electrophoresis.

Chloroquine Resistance Markers

PCR BUFFERS AND NUCLEOTIDE MIX

5X PCR BUFFERS

<u>BUFFER</u>	<u>ul 10x GIBCO buffer</u>	<u>ul 50mM MgCl₂</u>	<u>sterile distilled water</u>
5X CRT	500	150	350

5X CRT buffer contains 12.5 mM MgCl₂ (for final concentration 2.5 mM)

10X NUCLEOTIDE MIX

Take 26ul each of 100mM dNTP(G,A,T and C) into 1196ul of 1X TE

This 10X mix of nucleotide is 2mM for each nucleotide

Taq DNA polymerase

Use at 2.5u per 100ul reaction volume

Primers. PCR primers are generally used at a final concentration of 1uM, and prepared at a 100X concentration of 100uM in TE buffer.

Primer concentration determination with spectrophotometer

We generally synthesize our primers from the in-house biopolymer lab in 1.0 umole quantities divided into two 1.5ml centrifuge tubes. Resuspend one tube in 500 ul TE buffer, mix thoroughly and allow to dissolve for at least 10 min. Add 2 ul of primer to a UV spec cuvette containing 1 ml distilled water, mix manually, and determine A₂₆₀. The concentration can be calculated using the following equation:

$$\text{ug/ml primer} = 500 \times 33 \times A_{260}.$$

To convert mass to molarity, the following equations are helpful.

1 ug of 1 fb (*P. falciparum* base) = 3.5 nmol fb.

Thus, for a 17 -mer oligonucleotide, 1 ug = 3.5/17 = 0.2nmol 17 -mer.

If stock primer c. is 5mg/ml , it is also 5 ug/ul and thus 5x0.2 or 1nmol/ul, that is, 1mM.

Therefore, **mM primer = (3.5/n) x C** , where n = #nucleotides/primer, and C = primer c. in g/ml.

Nested PCR and restriction digestion methods for the detection of *pfcrt* K76T in field isolates.

1-3 ul of the amplicon from the PCR1 described above is amplified during a second nested PCR using inner primers around the K76T mutation. These primers:

CRTD1 = TGTGCTCATGTGTTTAACTT
 CRTD2 = CAAAACATATAGTTACCAATTTTG

amplify a 134 bp segment around the 76 mutation. Cycling parameters are:

- ? initial denaturation at 95°C for 5min
- ? 25 – 30 cycles of
- ? denaturation at 92°C for 30s
- ? annealing at 48°C for 30s
- ? extension at 65°C for 30s
- ? final extension at 65°C for 3min

MgCl₂ is present at a concentration of 2.5mM, with a 200µM concentration of each dNTP and a 1µM concentration of each primer.

5ul of the amplicon from the second PCR is cut with 0.5 U of the restriction enzyme *Apo1* during a 6H incubation at 50°C according to the manufacturer's protocol. The digest is run onto a 2% agarose gel containing ethidium bromide for at least 1 Hour at 95V. *Apo1* cuts out 34 bp from the wild type allele but does not cut the mutant one. Positive and negative controls are included at all steps as described above.

Nested PCR methods for the detection of *pfcr* polymorphism

Mutation-specific PCR.

In the first step, flanking primers amplifying a 537bp region around the mutation K76T are used. The primers:

CRTP1 = CCGTTAATAATAAATACACGCAG
CRTP2= CGGATGTTACAAAACATAGTTACC

are used with the following cycling parameters:

- ? initial denaturation at 94°C for 3 min
- ? 45 cycles of
- ? denaturation at 94°C for 30s
- ? annealing at 56 °C for 30s
- ? extension at 60 °C for 1min
- ? final extension at 60°C for 3 mn.

MgCl₂ is present at a concentration of 2.5mM, with a 200µM concentration of each dNTP and a 1µM concentration of each primer.

Genomic DNA extracted by the standard chloroform-phenol method from the laboratory-adapted chloroquine-resistant strain Dd2 and chloroquine-sensitive strain 3D7, are used as positive controls. Water is used as negative control. The product is run on a 2% agarose gel. Field samples may not yield a visible band at this step, but as long as the positive controls yield bands one should proceed with the second PCR.

In the second step, mutation-specific diagnostic PCR is performed. 1-3 ul of the amplicon of the first PCR reaction is amplified using 1uM of a common inner primer:

CRTP3 = TGACGAGCG TTATAGAG

coupled with 1uM of one of the allele specific primers:

CRTP4m = GTTCTTTTAGCAAAAATTG or
CRTP4w = GTTCTTTTAGCAAAAATcT

which detect the mutant allele (resistant) or the wild type allele (sensitive), respectively. The cycling conditions for the second step are:

- ? initial denaturation at 94°C for 3min
- ? 15 – 30 cycles of
- ? denaturation at 94°C for 30s
- ? annealing at 47°C for 30s
- ? extension at 64°C for 1min
- ? final extension at 64°C for 3mn

MgCl₂ is present at a concentration of 1.5mM, with a 200 ?M concentration of each dNTP and a 1 ?M concentration of each primer. Amplicons from the positive control reactions in the first step are used as positive controls. Two negative controls are used: the amplicon of the negative control for the first PCR and a new water control. Each set of primers (CRTP3 + CRTP4m or CRTP3 + CRTP4w) is used in a separate tube and the resulting amplicon loaded into two consecutive wells on a 2% agarose gel containing ethidium bromide. Samples with the mutant allele or the wild type allele will yield a 366bp band from the corresponding well while samples with mix infections will yield a band in both wells and negative samples should yield no bands.

Agarose gel casting

Procedure	Comments
1. Seal gel tray with autoclave tape and place it on a horizontal surface. The slot-forming combs should be positioned now.	Place the tray in a cold slab. This yields high transparency, and the agarose will gel after a few minutes
2. Mix agarose at the desired concentration (w/v) with 1 x electrophoresis buffer in an Erlenmeyer flask, or measuring cylinder. The gel suspension should not occupy more than 50% of the container	Agarose has to be suspended completely, prior to heating. This might necessitate vigorous shaking. The buffer for gel casting and electrophoresis has to be from the same batch. Recommended gel thickness, 3mm
3. Boil in a microwave oven or in a boiling water bath. Shake from time to time, take care: superheated gel may boil over spontaneously	
4. When the gel is completely dissolved add 1 µl ethidium bromide per 20 ml gel solution and mix thoroughly	The gel solution should be capped so that no "skin" forms at the cooling surface. Ethidium Bromide stock: 10mg/ml: final 0.5 µg/ml. Alternatively, stain the gel with buffer containing ethidium bromide solution (0.5 to 1.00 µg/ml) for 30 minutes. Destain the gel in a tray of water for another 10-15 minutes.
5. Cast the gel as hot as necessary (50 to 60°C), without pause, into prepared apparatus or tray. Tilt once so the gel flows behind the comb. Remove air bubbles with a pipette tip	
6. The gel will be ready for use after 10-30 minutes at RT. Submerge the gel in electrophoresis buffer before removing the combs.	

Note:: Weigh the appropriate amount of agarose (1.5% to 3%): note for the mixed agarose at 3:1 proportion {ultrapure agarose : NuSieve agarose}, this should be done in 3:1 ratio e.g., for 1.5% gel, weigh 1.125 g of ultrapure agarose and 0.375g of NuSieve). When using agarose alone, weigh 1.5 g for the 1.5% gel.

Sample Preparation for Electrophoresis

The loading buffer is made at 5X or 10X the final concentration, therefore, for each tube the amount of Loading buffer to be added is a quarter or one ninth of the volume of the PCR reaction OR [1/5 conc.] to [1/10 conc.].

Sucrose is added to the loading buffer in order to make the final mixture heavy, and thus possible to load in the wells of the gel. The Tris buffer keeps the pH at 8.0, an optimum value for DNA storage.

Three types of tracking dye can be used in combination or separately. These dyes are the only way to monitor the extent of electrophoresis, and thus to prevent sample loss by over-electrophoresis! The 3 dyes differ in their electrophoretic mobility, the fastest being Orange G, Bromophenol blue is slower and Xylene cyanole is the slowest. The extent of migration, as compared with DNA, depends on the type of electrophoretic matrix used. Ensure that the tracking dye does not migrate at the same level as the expected DNA product, since it may obscure the bands and prevent their detection, this is especially true with Bromophenol blue and Xylene cyanole dyes. Orange G migrates similarly to a DNA molecule of about 100bp, and is therefore unlikely to obscure amplification products.

The loading dye contains SDS and EDTA which will prevent any bacterial growth and will inhibit degradation of the amplification product.

Sample loading

Procedure	Comments
1. Preload 1 ?l of 10X Gel loading Buffer (GLB) into pre-labelled 0.5 ml eppendorf tubes corresponding to nest II labels.	
2. Pipet 9 ?l of sample without oil, remove oil with a lint-free paper towel and mix the sample well with the GLB.	The sample has to be mixed again before loading into the gel.
3. Load sample into slot	The gel has to be submerged in electrophoresis buffer before sample loading. Overload may cause contamination of neighbouring slot.
4. Start electrophoresis immediately.	Apply with direct current electricity at constant voltage of 100 volt for 1.5-2 hours.
5. Visualize and photograph the stained gel on the UV transilluminator	

Table 3: Restriction Enzymes Targets for Molecular Markers of Resistance by ASRA-PCR & RFLP-PCR Methods

Gene	Mutation	P	Primer Sequences	PCR conditions	Enzyme digest	Control DNA
Msp1 Primary		O1 O2	5' CACATGAAAGTTATCAAGAACTTGTG 3' 5' GTACGTCTAATTCATTTGCACG 3'	94°C-3min, 94°C-25sec, 50°C-45sec, 68°C-2min, x30, 72°C-3min, 4°C-hold		HB3, 3D7 W2
Msp1 Nested		N1 N2	5' GCAGTATTGACAGGTTATGG 3' 5' GATTGAAAGGTATTTGAC 3'	94°C-3min, 94°C-30sec, 50°C-45sec, 68°C-2min, x30, 72°C-3min, 4°C-hold		
Msp2 Primary		S3 S2	5' GAAGGTAATTAACATTGTG 3' 5' GAGGGATGTTGCTGCTCCACAG 3'	94°C-3min, 94°C-30sec, 42°C-60sec, 65°C-2min, x30, 72°C-3min, 4°C-hold		HB3, 3D7 W2
Msp2 Nested		S1 S4	5' GAGTATAAGGAGAAGTATG 3' 5' CTAGAACCATGCATATGTCC 3'	94°C-30sec, 50°C-60sec, 72°C-2min, x30, 72°C-3min, 4°C-hold		
GLURP Primary		G4 G5	5' ACATGCAAGTGTGATCC 3' 5' GATGGTTTGGGAGTAACG 3'	94°C-3min, 94°C-25sec, 45°C-60sec, 68°C-2min, x30, 72°C-3min, 4°C-hold		HB3, 3D7 W2
GLURP Nested		G1 G3	5' TGAATTCGAAGATGTTACACTGAAC 3' 5' TGTAGGTACCACGGGTTCTTGTG 3'	94°C-60sec, 55°C-2min, 70°C-2min, x30, 72°C-3min, 4°C-hold		
Pfprt Primary	K76T	CRTP1 CRTP2	5' CCGTTAATAATAATACACGCAG 3" 5'CGGATGTTACAAAACATATAGTTACC	94°C-3min, 94°C-30sec, 56°C-30sec, 60°C-1min, x45, 60°C-3min, 4°C-hold		
Pfprt Nested	K76T	CRTD1 CRTD2	5' TGTGCTCATGTGTTTAACTT 3' 5' CAAAACATATAGTTACCAATTTTG.	95°C-5min, 92°C-30sec, 48°C-30sec, 65°C-30sec, x30, 65°C-3min, 4°C-hold	ApoI	(+) 3D7
Pimdr1 Primary	N86Y	MDR1 MDR2	5'GCGCGCGTTGAACAAAAAGAGTACCGCTG 3' 5'GGGCCCTCGTACCAATTCCTGAAC TCAC 3'	95°C-5min, 95°C-30sec, 45°C-30sec, 65°C-45sec, x45, 72°C-5min, 4°C-hold		
Pimdr1 Nested	N86Y	MDR3 MDR4	5' TTTACCGTTTAAATGTTTACCTGC 3' 5' CCATCTTGATAAAAAACACTTCTT 3'	95°C-3min, 95°C-30sec, 45°C-30sec, 65°C-45sec, x25, 72°C-5min, 4°C-hold	<u>AfIII</u>	(+) 7G8
Dhfr Primary		M1 M5	5' TTTATGATGGAACAAGTCTGC3' 5' AGTATATACATCGCTAACAGA3'	94°C-3min, 94°C-1min, 50°C-2min, 72°C-2min, x40, 72°C-10min, 4°C-hold		
	A51C	M3 F/			Tsp5091	(+) K1
	R59C	M3 F/			XmnI	(+) T9/96
	S108N S108T	M3, F/ F M4	5'GAAATGTAATTCCCTAGATATGgAAT ATT3' 5'TTAATTTCCCAAGTAAACTATTAGA gCTTC3	94°C-2min, 94°C-1min, 45°C-1min, 72°C-2min, x35, 72°C-10min, 4°C-hold	AluI BsrI BstNI	(+) HB3 (+) T9/96 (+) FCR3
	I164L	M3 F/			Dra I	(+) V1/S
Dhps Primary		R2 R/	5' AACCTAAACGTGCTGTTCAA3' 5' AATTGTGTGATTGTCCACAA3'	94°C-3min, 94°C-1min, 50°C-2min, 72°C-2min, x40, 72°C-10min, 4°C-hold		
Dhps Nested	A437G	K K/	5'TGCTAGTGTTATAGATATAGGatGAG cATC3' 5'CTATAACGAGGTATTgCATTTAATgC AAGAA3'	94°C-2min, 94°C-1min, 45°C-1min, 72°C-2min, x35, 72°C-10min, 4°C-hold	MwoI / AvaII	(+) K1
	K540D	K K/			Fok I	(+) TN-1

Quality Assurance

Glossary

<i>Assessment</i>	<i>Assessment procedures to monitor the analytical process to determine the type and amount of error and change in accuracy and precision</i>
<i>Quality Assurance:</i>	<i>coordinated effort designed to detect and prevent the occurrence of errors in a laboratory</i>
<i>Sensitivity</i>	<i>the least amount of biological material detectable by the system</i>
<i>Sensitivity</i>	<i>refers to the highest proportion of true positives that an assay can pick up in a survey</i>
<i>Quality Control:</i>	<i>The process of monitoring the accuracy and precision of a laboratory test with control samples</i>
<i>Accuracy:</i>	<i>The measure of correctness or truth of a laboratory test.</i>
<i>Precision:</i>	<i>The measure of variability in laboratory test process.</i>

Each laboratory will ensure that tests are done with precision, and properly analyzed, that errors are detected on time and corrected and that further errors are prevented in a timely manner. Quality assurance is not a single activity nor the responsibility of a single individual but must be practiced by everyone within the laboratory. It involves different components listed below:

- 1) *Preventive activities*, (prevent errors and to improve accuracy and precision). –
 - careful laboratory design,
 - hiring of competent personnel,
 - adoption of standard operating procedures
 - preventive maintenance programs
 - ensuring safety procedures
- 2) *Assessment and Corrective actions*
 - quality control and instrument performance function checks
 - proficiency testing programs for personnel
 - timely communication with the users of services, regular review of laboratory notebooks and prompt trouble shooting
- 3) *Equipment Assessment and Maintenance*
 - All equipment and instruments must be checked and status reported
 - All pipettes must be maintained and calibrated every 6 months
 - Log books for equipment used must be provided

Quality Control

A. Laboratory-Based Assessment

1- Experimental Controls:

- Positive controls: ensures sensitivity of assay and includes nucleic acids with target sequences
- Negative Control 1(NC1): free of target sequences in primary amplification tests for non-contamination of reagents
- Negative Control 2(NC2): tests for non-specific amplification during primary PCR, is performed with an aliquot of the primary PCR.

2- Internal Control:

When a PCR does not yield bands or amplify - perform an internal control.

- Spike a specified or known amount of positive genomic nucleic acid sequence containing the known primer binding sites during amplification alongside a tube with suspected negative result DNA
- Alternatively include the DNA at the start of extraction and repeat PCR procedures.

B Network Based Assessment

3- Internal Quality Assessment

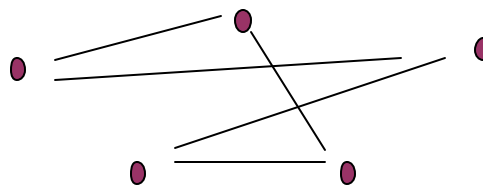
Tests the precision of a system at specific intervals – (MR4 - November)

- *Samples will be sent to you that contain positive and negative samples mixed in different proportions and different DNA amounts for performance of the PCR.*
- *These results will be used to determine a regional quality control with mean and standard deviation etc*

4- External Quality Control:

Compares results between laboratories and measures the accuracy of the test method.

- *Each lab will send to the Network Manager 10 random filter paper samples per 100 collected from another laboratory or site. Different network laboratories will get to analyse different samples from others as shown below.*



5- Proficiency Survey:

- Identical 50 different samples will be sent to each laboratory for analysis
- Samples will be analysed and compared. The accuracy of a laboratory's methods can be derived by comparing its results to the sample mean and standard deviation. The closer the result to the mean, the more accurate that laboratory's determination.

Requirements for molecular markers analysis

Sample collection -:	3M Whatman filter paper, lancets, alcohol, microscope
DNA extraction:	Microcentrifuge, vortex mixer, pipette set, water bath, plasticware, reagents, methanol and chelex
PCR:	Thermocycler, plasticware, PCR set of three pipettes, reagents, dNTPs, oil, restriction enzymes, primers.
Analysis:	Gel electrophoresis mold, tanks and combs, power packs, agarose, transilluminators, analysis pipette set, reagents (size markers), gel documentation /digital camera, hood, computer, printer and zip drive/

Data Management

Capacity to efficiently store, transfer, retrieve, analyse, relate, and collate large amount of data in a secure manner is critical to the success of the network and accomplishment of the set objectives. It is essential that the analysis of the data set is done globally to provide information that will address the definition of anti-malaria drug resistance in a scientific manner. A web based data storage system will be developed and will incorporate other modules of teaching and general interest including discussion groups. The web site will have appropriate security checks and restrictions for members of the public, to prevent access of non network investigators. However network investigators with appropriate security clearance will be able to upload data and review data with information from the other sites. Investigators would upload status on data collection monthly on the web sites. The investigators will continue to explore modalities for the publication of the network and site specific data.

The network will adopt Microsoft access as the database entry platform.